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Molecular analyses of shallow-water zooxanthellate zoanthids (Cnidaria: Hexacorallia) from Taiwan and their *Symbiodinium* spp

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Abstract

Background: Although we previously reported on surveys of shallow-water zoanthid (Anthozoa: Hexacorallia: Zoantharia) species diversity from localities in Taiwan, no study has yet examined the molecular phylogeny of the majority of specimens, nor has any study examined zoanthid zooxanthellae (*Symbiodinium* spp.) diversity in Taiwan. As many phylogenetic results have been reported from Japan, a critical step in testing DNA identification of zoanthids and their symbionts is the application of these techniques to specimens from neighboring regions such as Taiwan. We utilized three DNA markers (mitochondrial cytochrome oxidase subunit I (COI), mitochondrial 16S ribosomal DNA (mt 16S rDNA), and the nuclear internal transcribed spacer region of ribosomal DNA (ITS-rDNA)) to examine the molecular phylogeny of 110 zooxanthellate specimens collected from 10 localities in Keelung, Kenting, and Green Island. Additionally, we report on the types of *Symbiodinium* spp. (zooxanthellae) from the three regions above plus the Penghu Islands, utilizing sequences of ITS2-rDNA.

Results: Most specimens in the suborder Brachycnemina belonged to previously reported species. However, it appears that there are still some undescribed *Palythoa* species groups in the northwestern Pacific that are cryptic in having small colony sizes (<5 polyps) and occurring in low numbers. With regards to *Symbiodinium* spp. diversity, *Palythoa tuberculosa* and *Palythoa mutuki* associated with subclade C1 and related types, *Zoanthus sansibaricus* associated with subclade C3-derived types, and *Zoanthus kuroshio* that harbored C15/C91-related *Symbiodinium*. Notably, 1/2 of the *P. tuberculosa* specimens from the Penghu Islands harbored a previously unreported clade C symbiont, and two *Zoanthus. vietnamensis* specimens from Keelung also had a unique symbiont type within clade C.

Conclusions: It appears that undescribed *Palythoa* species in the northwestern Pacific may be widely distributed yet low in numbers, often cryptic in their habitat, and with a small colony size. As well, it is clear that much work remains to understand zoanthid-symbiont associations in Taiwan.

Keywords: Marine biodiversity; COI; mt 16S rDNA; ITS-rDNA; Taiwan; Zoanthid; Zooxanthellae

Background

Zoanthids are an order (Zoantharia = Zoanthidea) of Hexacorallia (Cnidaria: Anthozoa) and are common benthos in tropical and subtropical shallow waters (e.g., Karlson 1980). Species of the zooxanthellate genera *Zoanthus* (family Zoanthidae) and *Palythoa* (Sphenopidae) within the suborder Brachycnemina are usually dominant components of live reef cover, particularly on coral reef tops and reef edges (Burnett et al. 1997; Swain 2010; Irei et al. 2011). Nevertheless, species identification in both genera is problematic due to much intraspecific variation and plasticity (Burnett et al. 1994, 1997; Reimer et al. 2004; Ong et al. 2013) and a high "synonymy load" due to inadvertent redescriptions of the same species from different localities (Burnett et al. 1997).

Recent examinations of zoanthids using phylogenetic methods have fostered a new understanding of their species diversity and evolutionary relationships (Burnett et al. 1994, 1997; Reimer et al. 2004; Sinniger et al. 2005, 2013; Swain 2010), and much research has focused on zoanthids in Japanese waters in the northwestern Pacific (e.g., Reimer et al. 2006a,c, 2007c). In Taiwan, recent

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work using morphological field identification resulted in a brachycnemic zoanthid species list for the region (Reimer et al. 2011b), but specimens (excepting *Acrozoanthus* in Reimer et al. 2010) have not yet been examined using molecular methods.

Many brachycnemic zoanthids are in symbioses with endosymbiotic dinoflagellates of the genus *Symbiodinium* (= zooxanthellae). Not only zoanthids, but also many scleractinian corals, actiniarians, giant clams, and foraminifers are known to harbor *Symbiodinium*, and zooxanthellate organisms are therefore responsible for creating much of the architecture of coral reef ecosystems. Based on molecular phylogenetic studies, *Symbiodinium* is currently divided into nine clades of unknown taxonomic rank (Pochon and Gates 2010), and there are numerous subclades with different associated host species within each clade (e.g., LaJeunesse 2002, 2005; LaJeunesse et al. 2003, 2004), each potentially having different physiologies (Tchernov et al. 2004). For these reasons, research on *Symbiodinium* diversity is therefore needed to clearly understand the diversity of coral reef and shallow-water ecosystems, and also help anticipate what changes may occur under the threat of global warming.

Recent work on *Symbiodinium* in brachycnemic zoanthids in Japan revealed different levels of specificity in symbiont-host relationships. Species of the genus *Palythoa* (family Sphenopidae) generally associate with subclade C1-related *Symbiodinium* in Japan and the Indian Ocean, and with clade D in the Indian Ocean (Burnett 2002, Reimer et al. 2006d, Reimer and Todd 2009). However, there appears to be greater variation within the widely distributed species *Zoanthus sansibaricus* (family Zoanthidae), which is known in the Ryukyu Islands to be associated with subclade A1 in intertidal areas with high light levels, with subclade C1z sensu Reimer et al. (2007b) in most areas (Reimer et al. 2006e, 2007b), and with another subclade (related to C1) at >7 m in depth (Kamezaki et al. 2013).

In this study, we examined (1) zooxanthellate, shallow-water zoanthid species of Taiwanese waters first reported in Reimer et al. (2011b), this time utilizing molecular techniques and discuss the implications of our results and (2) *Symbiodinium* of the same zoanthid specimens plus of additional new specimens from the Penghu Islands in the Taiwan Strait. Taiwan, while close to Okinawa, has a variety of subtropical and tropical environments that differ from Okinawa, and there is the possibility of differing symbiont associations. For example, the Keelung region of northeastern Taiwan is much cooler in winter than waters in Okinawa, and more reminiscent of environments along the Pacific coast of mainland Japan (Chen 1999; Chen and Shashank 2009), while areas of Green Island off southeastern Taiwan and the coastal area of Kenting (southern Taiwan) have tropical coral reefs. The Penghu Islands, to the west of the main island of Taiwan, are heavily influenced by seasonal monsoons, and waters are often turbid (Huang 2012a, b). Therefore, knowledge of zoanthid-symbiont associations in Taiwan can help expand upon various recent observations of zoanthid-symbiont symbioses from Japan.

Methods

Surveys of zoanthids in Taiwan were carried out between September 2009 and September 2010 by snorkeling and scuba diving, with additional surveys in December 2011 and September 2012. Twelve investigated sites were divided over four different regions: (1) Kenting, southern Taiwan (Wanlitung, Tiaoshi, Hobihoo, and Longkeng sites; sampling permission #989094/1400); (2) Green Island (*Lyudao* in Chinese), off the southeastern coast of Taiwan (Gueiwan, Zolo, Shihlang, Dabaisha, and Matichao sites); (3) Keelung, northeastern Taiwan (Yeliu and Bitouchiao), and (4) the Penghu Islands off the west coast of Taiwan in the Taiwan Strait (Wangan) (Table 1). Some of these surveys and specimens were previously reported in Reimer et al. (2011b). The Keelung region primarily consists of subtropical non-reefal coral communities (Chen 1999; Chen and Shashank 2009), while the other two regions are coral reef ecosystems. In total, 110 specimens were collected (Table 1).

Specimens were photographed *in situ* prior to collection for characterization of external morphological features (oral disc diameter, color and patterns, and tentacle numbers). Identification of all zooxanthellate species followed Reimer (2010) and Reimer et al. (2011b) except for *Acrozoanthus* specimens identified following Ryland (1997) and Reimer et al. (2011a). All specimens were identified as in Reimer et al. (2011b) unless otherwise noted (see "Results"). Results for the formally undescribed and azooxanthellate *Palythoa* sp. "tokashiki" as reported in Reimer et al. (2011b) will be separately reported when this species is formally described.

Specimens were preserved in 70% or 99.5% ethanol, and examined at the Univ. of the Ryukyus (UR). Currently, specimens are deposited at the 1st author's laboratory at the UR, but once additional examinations are completed, specimens will be deposited at the National Museum of Natural Science (NMNS), Taichung, Taiwan.

DNA extraction and polymerase chain reaction (PCR) amplification

DNA was extracted from specimens either with a guanidine method described by Sinniger et al. (2010) or using a spin column DNeasy Blood & Tissue kit (Qiagen, Tokyo, Japan) following the manufacturer's protocol. The PCR amplification of the extracted genomic DNA was performed using a HotStarTaq Plus Master Mix Kit (Qiagen) following the manufacturer's instructions. Three

Table 1 Identity of zoanthid specimens from Taiwan examined in this study, their collection information, and associated GenBank accession numbers

Specimen number	Location	Region	Date*	Depth (m)	Collector	mt 16S rDNA	COI	ITS-rDNA	*Symbiodinium* ITS-rDNA	Identity
1582	Wanli Tung	Kenting	04-Sep-09	4	JD Reimer	KF499618	NA	NA	NA	*Palythoa tuberculosa*
1583	Wanli Tung	Kenting	04-Sep-09	4	JD Reimer	KF499617	NA	NA	NA	*P. tuberculosa*
1584	Wanli Tung	Kenting	04-Sep-09	4	JD Reimer	KF499616	NA	NA	NA	*P. tuberculosa*
1585	Wanli Tung	Kenting	04-Sep-09	5	JD Reimer	NA	NA	NA	NA	*P. tuberculosa*
1586	Wanli Tung	Kenting	04-Sep-09	5	JD Reimer	KF499615	NA	NA	KF499805	*P. tuberculosa*
1587	Wanli Tung	Kenting	04-Sep-09	4	JD Reimer	KF499666	KF499698	NA	NA	*P. tuberculosa*
1588	Wanli Tung	Kenting	04-Sep-09	2	JD Reimer	KF499665	KF499699	NA	NA	*P. tuberculosa*
1589	Wanli Tung	Kenting	04-Sep-09	1	JD Reimer	KF499667	KF499706	NA	NA	*Zoanthus vietnamensis*
1590	Wanli Tung	Kenting	04-Sep-09	1	JD Reimer	KF499668	KF499705	NA	NA	*Z. vietnamensis*
1591	Wanli Tung	Kenting	04-Sep-09	1	JD Reimer	NA	NA	NA	NA	*Z. sansibaricus*
1592	Wanli Tung	Kenting	04-Sep-09	1	JD Reimer	NA	NA	NA	NA	*Z. sansibaricus*
1593	Wanli Tung	Kenting	04-Sep-09	1	JD Reimer	NA	NA	NA	NA	*Z. sansibaricus*
1594	Wanli Tung	Kenting	04-Sep-09	2	JD Reimer	KF499663	KF499695	NA	KF499794	*Palythoa* sp. "sakurajimensis"
1595	Wanli Tung	Kenting	04-Sep-09	2	JD Reimer	KF499661	KF499697	NA	KF499816	*Palythoa* sp. "sakurajimensis"
1596	Wanli Tung	Kenting	04-Sep-09	2	JD Reimer	NA	KF499704	NA	NA	*Palythoa mutuki*
1597	Wanli Tung	Kenting	04-Sep-09	1	JD Reimer	KF499	KF499696	KF499778	NA	*Palythoa* sp. "sakurajimensis"
1598	Wanli Tung	Kenting	04-Sep-09	NA	K Shashank	KF499620	NA	NA	KF499806	*P. tuberculosa*
1599	Wanli Tung	Kenting	04-Sep-09	NA	K Shashank	KF499619	KF499693	NA	KF499807	*P. tuberculosa*
1600	Tiao-shi	Kenting	04-Sep-09	8	JD Reimer	KF499621	KF499692	NA	NA	*P. tuberculosa*
1601	Tiao-shi	Kenting	04-Sep-09	6	JD Reimer	KF499622	KF499691	NA	NA	*P. tuberculosa*
1602	Tiao-shi	Kenting	04-Sep-09	5	JD Reimer	KF499623	KF499690	NA	NA	*P. tuberculosa*
1603	Tiao-shi	Kenting	04-Sep-09	5	JD Reimer	KF499624	KF499689	NA	NA	*P. tuberculosa*
1604	Tiao-shi	Kenting	04-Sep-09	5	JD Reimer	KF499625	KF499688	NA	NA	*P. tuberculosa*
1605	Tiao-shi	Kenting	04-Sep-09	5	JD Reimer	NA	NA	NA	NA	*P. tuberculosa*
1606	Tiao-shi	Kenting	04-Sep-09	5	JD Reimer	NA	NA	NA	NA	*P. tuberculosa*
1616	Ho-bi-hoo	Kenting	04-Sep-09	9	JD Reimer	KF499626	KF499687	NA	NA	*P. tuberculosa*
1617	Yeliu	Keelung	06-Sep-09	1	JD Reimer	KF499627	KF499745	KF499767	KF499787	*P. mutuki*
1618	Yeliu	Keelung	06-Sep-09	2	JD Reimer	KF499638	KF499744	KF499764	NA	*P. mutuki*
1619	Yeliu	Keelung	06-Sep-09	2	JD Reimer	KF499637	KF499743	NA	KF499827	*P. mutuki*
1620	Yeliu	Keelung	06-Sep-09	1	JD Reimer	KF499636	KF499742	NA	KF499826	*P. mutuki*
1621	Yeliu	Keelung	06-Sep-09	1	JD Reimer	KF499635	KF499741	KF499765	KF499786	*P. mutuki*
1622	Yeliu	Keelung	06-Sep-09	2	JD Reimer	KF499646	KF499725	NA	KF499825	*Zoanthus gigantus*
1623	Yeliu	Keelung	06-Sep-09	2	JD Reimer	KF499634	KF499740	KF499766	NA	*P. mutuki*
1624	Yeliu	Keelung	06-Sep-09	2	JD Reimer	NA	NA	NA	NA	*P. mutuki*
1625	Yeliu	Keelung	06-Sep-09	2	JD Reimer	KF499648	KF499726	NA	KF499824	*Z. gigantus*
1626	Yeliu	Keelung	06-Sep-09	2	JD Reimer	NA	NA	NA	NA	*Z. gigantus*
1627	Yeliu	Keelung	06-Sep-09	2	JD Reimer	KF499678	KF499719	NA	KF499823	*Z. vietnamensis*
1628	Yeliu	Keelung	06-Sep-09	2	JD Reimer	KF499647	KF499724	NA	NA	*Z. gigantus*
1629	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499633	KF499739	NA	NA	*P. mutuki*

Table 1 Identity of zoanthid specimens from Taiwan examined in this study, their collection information, and associated GenBank accession numbers *(Continued)*

1630	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499632	KF499738	KF499768	KF499822	*P. mutuki*
1631	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499660	KF499737	KF499772	KF499785	*P. tuberculosa*
1632	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499650	KF499723	NA	KF499821	*Z. sansibaricus*
1633	Bitouchiao	Keelung	06-Sep-09	4	JD Reimer	KF499644	KF499718	NA	KF499784	*Z. gigantus*
1634	Bitouchiao	Keelung	06-Sep-09	8	JD Reimer	KF499659	KF499736	NA	NA	*P. tuberculosa*
1635	Bitouchiao	Keelung	06-Sep-09	8	JD Reimer	KF499652	KF499735	KF499776	KF499783	*Palythoa* sp. "sakurajimensis"
1636	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499643	KF499717	NA	NA	*Z. gigantus*
1637	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499677	KF499716	NA	KF499789	*Z. vietnamensis*
1638	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499676	KF499715	NA	KF499790	*Z. vietnamensis*
1639	Bitouchiao	Keelung	06-Sep-09	2	JD Reimer	KF499675	KF499714	NA	KF499791	*Z. vietnamensis*
1640	Bitouchiao	Keelung	06-Sep-09	3	JD Reimer	KF499658	KF499734	KF499774	KF499782	*P. tuberculosa*
1641	Bitouchiao	Keelung	06-Sep-09	3	JD Reimer	KF499657	KF499733	KF499775	KF499798	*P. tuberculosa*
1642	Yeliu	Keelung	06-Sep-09	Intertidal	JD Reimer	KF499639	NA	KF499769	KF499799	*P. mutuki*
1654	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499673	KF499763	NA	KF499801	*Zoanthus kuroshio*
1655	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499672	KF499762	NA	KF499788	*Z. kuroshio*
1656	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499656	KF499761	KF499773	NA	*P. tuberculosa*
1657	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499671	KF499760	NA	KF499793	*Z. kuroshio*
1658	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499631	KF499759	NA	KF499800	*P. mutuki*
1659	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499655	KF499758	NA	NA	*P. tuberculosa*
1660	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499653	KF499757	NA	NA	*P. tuberculosa*
1661	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499654	KF499756	NA	KF499803	*P. tuberculosa*
1662	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499674	KF499755	NA	KF499795	*Z. kuroshio*
1663	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499649	KF499754	NA	NA	*Z. sansibaricus*
1664	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499642	KF499753	NA	NA	*Z. gigantus*
1665	Zolo	Green I.	06-Sep-10	1	JD Reimer	NA	KF499752	NA	NA	*Z. gigantus*
1666	Zolo	Green I.	06-Sep-10	1	JD Reimer	NA	KF499751	NA	NA	*Z. gigantus*
1667	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499630	KF499750	NA	KF499808	*P. mutuki*
1668	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499679	KF499749	NA	NA	*Z. vietnamensis*
1669	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499629	KF499748	NA	KF499804	*P. mutuki*
1670	Zolo	Green I.	06-Sep-10	1	JD Reimer	KF499628	KF499747	NA	KF499809	*P. mutuki*
TF1	Shihlang	Green I.	01-Nov-09	7	T Fujii	NA	KF499731	NA	NA	*Parazoanthus* sp.
TF3	Dabaisha	Green I.	02-Nov-09	11	T Fujii	NA	NA	NA	NA	*Palythoa* sp.
TF4	Dabaisha	Green I.	02-Nov-09	10	T Fujii	NA	NA	NA	NA	*Acrozoanthus australiae*
TF5	Dabaisha	Green I.	02-Nov-09	10	T Fujii	NA	NA	NA	NA	*A. australiae*
TF6	Dabaisha	Green I.	02-Nov-09	10	T Fujii	NA	NA	NA	NA	*Palythoa* sp.
TF7	Dabaisha	Green I.	02-Nov-09	8	T Fujii	NA	NA	NA	NA	*Palythoa* sp.
TF8	Dabaisha	Green I.	02-Nov-09	4	T Fujii	KF499664	KF499732	GET	KF499813	*P. tuberculosa*
TF9	Dabaisha	Green I.	02-Nov-09	2	T Fujii	KF499645	KF499713	NA	NA	*Z. gigantus*
TF10	Dabaisha	Green I.	02-Nov-09	Intertidal	T Fujii	KF499651	KF499712	NA	GET(A)	*Z. sansibaricus*
TF11	Matichao	Green I.	02-Nov-09	16	T Fujii	NA	KF499728	NA	NA	*Epizoanthus illoricatus*
TF12	Matichao	Green I.	02-Nov-09	13	T Fujii	KF499612	KF499703	KF499771	KF499814	*Palythoa heliodiscus*

Table 1 Identity of zoanthid specimens from Taiwan examined in this study, their collection information, and associated GenBank accession numbers *(Continued)*

TF13	Matichao	Green I.	02-Nov-09	6	T Fujii	NA	NA	NA	NA	*Palythoa* sp.
TF14	Matichao	Green I.	02-Nov-09	3	T Fujii	KF499670	KF499727	NA	KF499797	*Z. vietnamensis*
TF15	Dabaisha	Green I.	02-Nov-09	1	T Fujii	KF499640	KF499746	NA	KF499815	*P. mutuki*
TF16	Matichao	Green I.	02-Nov-09	1	E Hirose	KF499669	KF499711	NA	KF499802	*Z. vietnamensis*
TF17	Gweiwan	Green I.	04-Nov-09	NA	T Fujii	KF499686	KF499722	NA	NA	*Hydrozoanthus gracilis*
TF20	Gweiwan	Green I.	04-Nov-09	21	T Fujii	NA	NA	KF499781	NA	*Palythoa* sp.
TF21	Gweiwan	Green I.	04-Nov-09	20	T Fujii	NA	NA	NA	NA	*Palythoa* sp.
TF22	Gweiwan	Green I.	04-Nov-09	8	T Fujii	NA	NA	NA	NA	*Palythoa* sp.
YI1	Shihlang	Green I.	01-Nov-09	2	Y Irei	KF499681	KF499708	NA	KF499796	*Z. vietnamensis*
YI2	Shihlang	Green I.	01-Nov-09	5	Y Irei	KF499614	KF499702	KF499779	KF499810	*P. heliodiscus*
YI3	Shihlang	Green I.	01-Nov-09	8	Y Irei	NA	NA	NA	NA	*Z. gigantus*
YI4	Shihlang	Green I.	01-Nov-09	9	Y Irei	KF499613	KF499701	KF499780	NA	*P. heliodiscus*
YI5	Shihlang	Green I.	01-Nov-09	9	Y Irei	NA	KF499730	NA	NA	*Parazoanthus* sp.
YI6	Shihlang	Green I.	01-Nov-09	7	Y Irei	NA	NA	NA	NA	*P. tuberculosa*
YI7	Shihlang	Green I.	01-Nov-09	9	Y Irei	NA	KF499729	NA	NA	*Parazoanthus* sp.
YI8	Dabaisha	Green I.	02-Nov-09	10	Y Irei	KF499685	KF499720	KF499777	KF499811	*Palythoa* sp. "sakurajimensis"
YI10	Dabaisha	Green I.	02-Nov-09	11	Y Irei	NA	NA	NA	NA	*H. gracilis*
YI11	Dabaisha	Green I.	02-Nov-09	11	Y Irei	NA	NA	NA	NA	*H. gracilis*
YI12	Dabaisha	Green I.	02-Nov-09	11	Y Irei	NA	KF499721	NA	NA	*Parazoanthus* sp.
YI13	Dabaisha	Green I.	02-Nov-09	4	Y Irei	NA	NA	NA	NA	*P. tuberculosa*
YI14	Dabaisha	Green I.	02-Nov-09	4	Y Irei	KF499641	KF499700	KF499770	KF499812	*P. mutuki*
YI15	Dabaisha	Green I.	02-Nov-09	2	Y Irei	NA	NA	NA	NA	*Z. sansibaricus*
YI16	Matichao	Green I.	02-Nov-09	15	Y Irei	NA	NA	NA	NA	*E. illoricatus*
YI17	Matichao	Green I.	02-Nov-09	5	Y Irei	NA	NA	NA	NA	*Palythoa* sp.
YI18	Matichao	Green I.	02-Nov-09	5	Y Irei	NA	NA	NA	NA	*Palythoa* sp.
YI19	Gweiwan	Green I.	04-Nov-09	9	Y Irei	KF499682	KF499710	NA	NA	*A. australiae*
YI20	Gweiwan	Green I.	04-Nov-09	9	Y Irei	KF499684	KF499709	NA	GET (D)	*A. australiae*
YI21	Sankuaiyan	Green I.	04-Nov-09	20	Y Irei	NA	NA	NA	NA	*Palythoa* sp.
YI22	Dabaisha	Green I.	02-Nov-09	Intertidal	Y Irei	NA	NA	NA	NA	*P. mutuki*
YI23	Dabaisha	Green I.	02-Nov-09	Intertidal	Y Irei	NA	NA	NA	NA	*Z. sansibaricus*
YI24	Dabaisha	Green I.	02-Nov-09	Intertidal	Y Irei	KF499680	KF499707	NA	KF499792	*Z. vietnamensis*
WLT1	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499817	*P. tuberculosa*
WLT2	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499828	*P. tuberculosa*
WLT3	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499829	*P. tuberculosa*
WLT4	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499830	*P. tuberculosa*
WLT5	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499831	*P. tuberculosa*
WLT6	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499832	*P. tuberculosa*
WLT7	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499833	*P. tuberculosa*
WLT8	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499834	*P. tuberculosa*
WLT9	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499835	*P. tuberculosa*
WLT10	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499836	*P. tuberculosa*
WLT11	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499837	*P. tuberculosa*
WLT12	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499838	*P. tuberculosa*

Table 1 Identity of zoanthid specimens from Taiwan examined in this study, their collection information, and associated GenBank accession numbers *(Continued)*

WLT13	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499839	*P. tuberculosa*
WLT14	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499840	*P. tuberculosa*
WLT15	Wanli Tung	Kenting	Sept 2012	3 to 5	SY Yang	NA	NA	NA	KF499841	*P. tuberculosa*
TWLK1	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499818	*P. tuberculosa*
TWLK2	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499842	*P. tuberculosa*
TWLK3	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499843	*P. tuberculosa*
TWLK4	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499844	*P. tuberculosa*
TWLK5	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499845	*P. tuberculosa*
TWLK6	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499846	*P. tuberculosa*
TWLK7	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499847	*P. tuberculosa*
TWLK8	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499848	*P. tuberculosa*
TWLK9	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499849	*P. tuberculosa*
TWLK10	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499850	*P. tuberculosa*
TWLK11	Longkeng	Kenting	Dec 2011	3 to 5	CY Kuo	NA	NA	NA	KF499851	*P. tuberculosa*
GIGW1	Gweiwan	Green I.	Sept 2012	NA	Y Nozawa	NA	NA	NA	KF499820	*P. tuberculosa*
GIGW2	Gweiwan	Green I.	Sept 2012	NA	Y Nozawa	NA	NA	NA	KF499859	*P. tuberculosa*
GIGW3	Gweiwan	Green I.	Sept 2012	NA	Y Nozawa	NA	NA	NA	KF499860	*P. tuberculosa*
GIGW4	Gweiwan	Green I.	Sept 2012	NA	Y Nozawa	NA	NA	NA	KF499861	*P. tuberculosa*
GIGW5	Gweiwan	Green I.	Sept 2012	NA	Y Nozawa	NA	NA	NA	KF499862	*P. tuberculosa*
PHWA1	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499819	*P. tuberculosa*
PHWA2	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499856	*P. tuberculosa*
PHWA3	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499857	*P. tuberculosa*
PHWA4	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499858	*P. tuberculosa*
PHWA9g1	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499852	*P. tuberculosa*
PHWA9g2	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499853	*P. tuberculosa*
PHWA9g3	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499854	*P. tuberculosa*
PHWA9g4	Wangan	Penghu Is.	Sept 2012	3 to 5	HJ Hsieh, AC Chung	NA	NA	NA	KF499855	*P. tuberculosa*

*All specimens in 2011 and 2012 newly reported in this study, other specimens collected as previously reported in Reimer et al. (2011b). Specimens with no sequence data identified only by external morphology. NA, not acquired.

DNA markers for zoanthid specimens were amplified in this study: (a) mitochondrial cytochrome oxidase subunit I (COI), (b) mitochondrial 16S ribosomal DNA (mt 16S rDNA), and (c) nuclear internal transcribed spacer region of ribosomal DNA (ITS-rDNA), using primers and amplification conditions respectively reported in Reimer et al. (2007a), Sinniger et al. (2010), and Reimer et al. (2007c) and Swain (2009). The ITS-rDNA of *Symbiodinium* spp. was amplified in this study using previously reported primers (White et al. 1990, Rowan and Powers 1992, Hunter et al. 1997) and amplification conditions reported in Reimer et al. (2007b).

All amplified products were visualized by 1.0% or 1.5% agarose gel electrophoresis, and positive products were treated with shrimp alkaline phosphate (SAP; Takara, Shiga, Japan) and exonuclease I. Sequencing was performed by MacroGen Japan (Tokyo, Japan) and Fasmac (Tokyo, Japan).

Phylogenetic analyses

Novel sequences obtained in this study were deposited in GenBank (accession nos.: KF499612-KF499862). For the mt 16S rDNA analyses, only specimens of brachycnemic sequences (= families Zoanthidae and Sphenopidae)

were included in the analyses, and sequences from the Macroncnemina (= families Epizoanthidae and Hydrozoanthidae) were excluded to improve the resolution of resulting phylogenetic trees. For mitochondrial COI and 16S-rDNA alignments, sequences of Macrocnemina were utilized as outgroups, while for ITS-rDNA, an alignment of only *Palythoa* spp., was generated, owing to the high levels of divergence between sequences in *Zoanthus* + *Acrozoanthus* and problems acquiring accurate sequences for these groups. For ITS-rDNA sequences from *Zoanthus* + *Acrozoanthus* specimens, analyses were done by direct sequence comparison with BLAST (National Library of Medicine, Bethesda, Maryland, USA).

The obtained novel *Symbiodinium* ITS-rDNA sequences (Table 1) were aligned with previously obtained clade C *Symbiodinium* sequences (see the resulting phylogenetic tree), with *Symbiodinium* subclade C15/C91 and related sequences as outgroups.

The three zoanthid alignments (all zoanthid COI, all zoanthid mt 16S rDNA, and *Palythoa* ITS-rDNA) were constructed as described in Reimer et al. (2012), following previous alignments as guides. All alignments were inspected by eye, and ambiguous sites (ambiguous or double peaks, <2 sites/alignment) were removed prior to the analyses. Three zoanthid alignment datasets were generated: (1) an 'all-zoanthid' COI alignment with 497 sites of 135 sequences, (2) an 'all-zoanthid' 16S-rDNA alignment with 685 sites of 120 sequences, and (3) a *Palythoa* ITS-rDNA alignment with 772 sites of 55 sequences. A *Symbiodinium* alignment of 275 sites of 101 sequences was generated. All four alignments are available from the corresponding author upon request.

For the phylogenetic analyses, the same methods were independently applied. Alignments were analyzed using neighbor-joining (NJ) and maximum-likelihood (ML) tests. ML was performed using PhyML (Guindon et al. 2010) with an input tree generated by BIONJ under the general time-reversible model (Lanave et al. 1984) of nucleotide substitution incorporating a discrete gamma distribution (eight categories) (GTR+). The discrete gamma distribution and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (1,000 replicates) were made utilizing the same parameters as the individual ML tree. Distances were calculated using a Kimura's two-parameter model (Kimura 1980). Support for the NJ branches was tested by bootstrap analysis (Felsenstein 1985) of 1,000 replicates. CLC Free Workbench 3.2.2 (Aarhus, Denmark) was used for the NJ phylogenetic analyses (1,000 replicates).

Results

COI

New COI sequences were obtained for 77 specimens. In the resulting ML tree from the COI alignment, *Palythoa* and *Zoanthus* + *Acrozoanthus* each formed large clades with generally high bootstrap values (ML = 58%, NJ = 96%, and ML = 96%, NJ = 99%, respectively) (Figure 1). Macrocnemina sequences for the families Hydrozoanthidae and Parazoanthidae were basal to Brachycnemina.

Within the *Palythoa* clade, the species *P. heliodiscus* (sequences matching 100% with previously reported GenBank accession sequence AB219214; ML = 97%, NJ = 99%) and *P. tuberculosa* (sequences matching 100% with previous *P. tuberculosa* sequences, e.g., AB128896 + others) (ML ≤ 50%, NJ = ≤ 50%) formed subclades. A single sequence (1623) of a *P. mutuki* specimen was included in the *P. tuberculosa* subclade. The majority of *P. mutuki* + unidentified *Palythoa* specimen (1595, 1597, 1635, and YI8) sequences (matching 100% with AB128895 + others) formed a polytomy basal to *P. heliodiscus*. Very little (1-bp difference) or no variation in specimen sequences was seen within subclades. The sequence from specimen 1594 was derived from *P. tuberculosa*, and had a 1-bp difference from *P. tuberculosa* (e.g., AB128896 + others).

Within the *Zoanthus* + *Acrozoanthus* clade, four subclades were observed; one corresponding to *A. australiae* (ML = 68%, NJ = 74%; sequences matching 100% with HM171914), another to *Z. gigantus* (ML = 84%, NJ = 93%; sequences matching 100% with AB128893 + others), another to *Z. sansibaricus* (ML = 94%, NJ = 95%; sequences matching 100% with AB194031 + others), and another to *Z. kuroshio* and *Z. vietnamensis* (ML = 63%, NJ = 65%; sequences matching 100% with AB252668 + others).

mt 16S rDNA

New mt 16S rDNA sequences were obtained for 73 specimens. In the resulting ML tree from the mt 16S rDNA alignment, *Palythoa* and *Zoanthus* + *Acrozoanthus* each formed large clades (ML ≤ 50%, NJ = 90%, and ML = 99%, NJ = 90%, respectively) (Figure 2).

Within the *Palythoa* clade, species groupings were observed, although only *P. heliodiscus* (sequences matching 100% with previously reported DQ997844 + others) formed a well-supported single subclade (ML = 99%, NJ = 99%). Sequences from specimens YI8, 1595, 1597, and 1635 were identical to those of *Palythoa* sp. "sakurajimensis" sensu Reimer et al. (2007c) (DQ997863 and DQ997842). The sequence from 1594 was identical to sequence DQ997862 from another *Palythoa* sp. "sakurajimensis" specimen from Japan, and formed a subclade (ML = 70%, NJ = 62%). *Palythoa mutuki* sequences formed a large subclade (matching 99% to 100% with previously reported *P. mutuki* sequences, e.g., DQ997841 + others) (ML ≤50%, NJ = 63%) with a 1-bp difference within the group, corresponding to *P. mutuki* 1 and *P. mutuki* 2 as previously reported (Reimer et al. 2006c, 2007c). Sequences from *P. tuberculosa* were

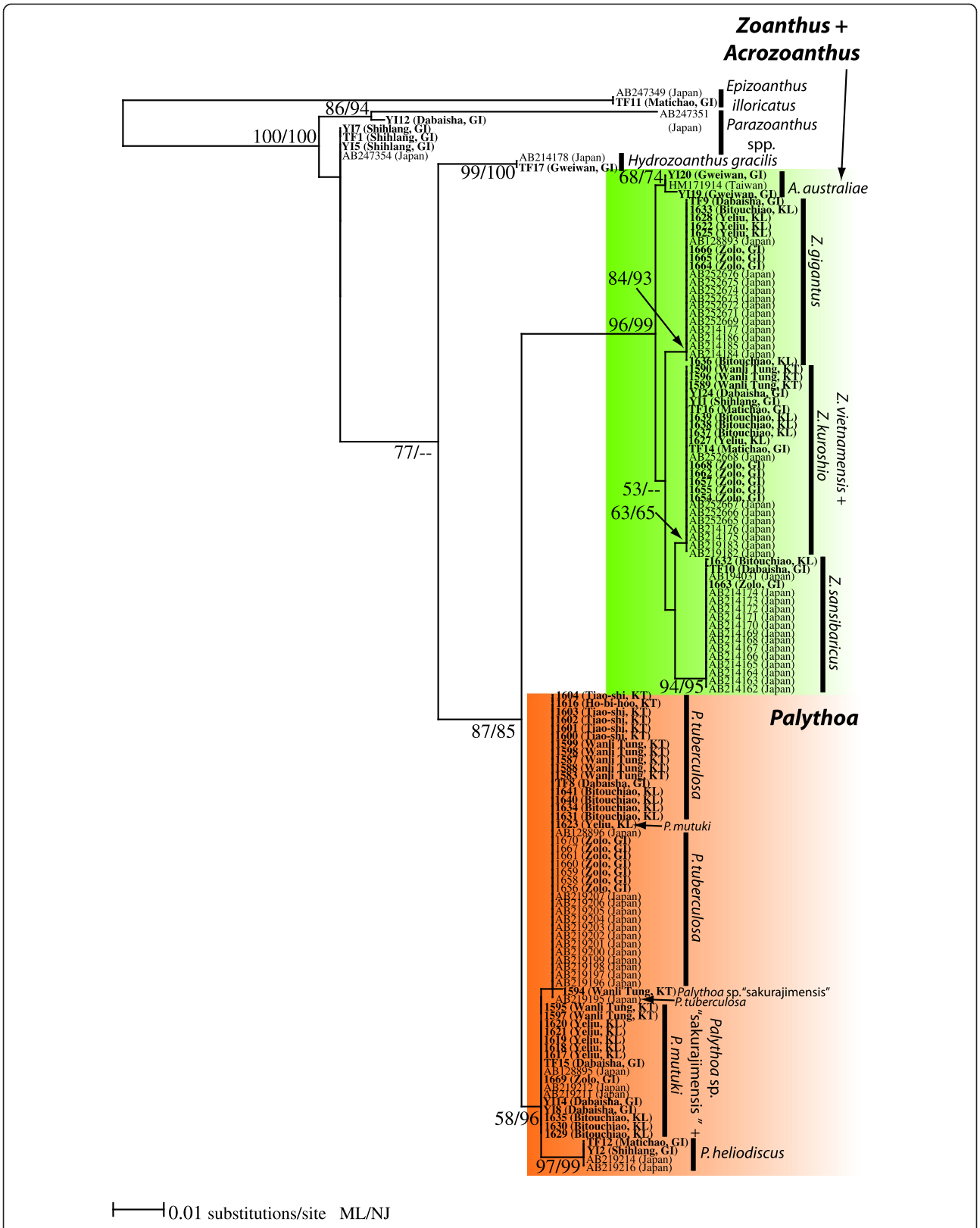

Figure 1 Maximum-likelihood (ML) trees of mitochondrial cytochrome oxidase subunit I sequences. Novel sequences from this study with specimen numbers and sampling locations are in boldface. Sequences from previous studies are included with GenBank accession numbers. Values at nodes respectively represent the ML and neighbor-joining (NJ) values. *KL*, Keelung; *KT*, Kenting; *GI*, Green Island.

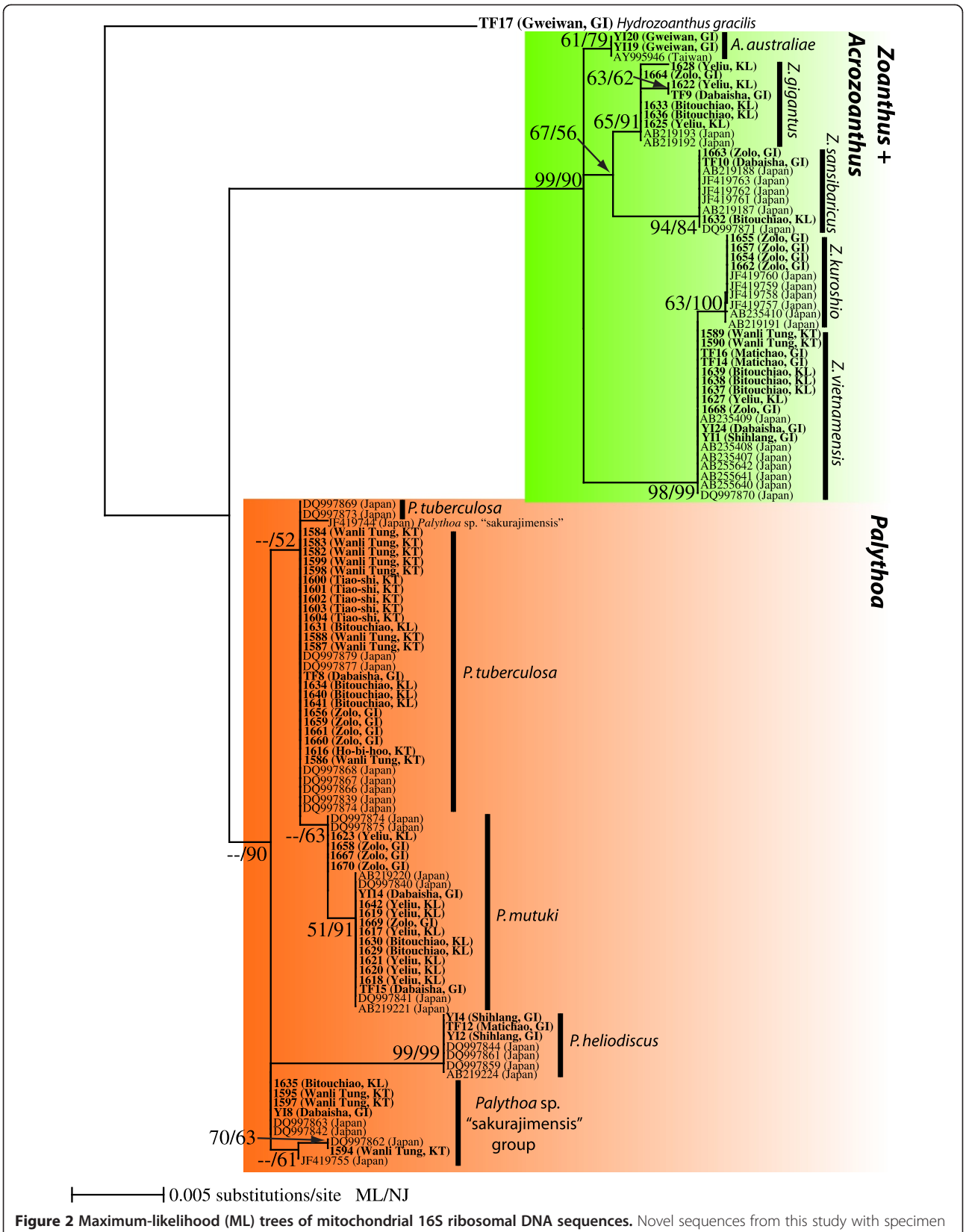

Figure 2 Maximum-likelihood (ML) trees of mitochondrial 16S ribosomal DNA sequences. Novel sequences from this study with specimen numbers and sampling locations are in boldface. Sequences from previous studies are included with GenBank accession numbers. Values at nodes respectively represent ML and neighbor-joining (NJ) values. *KL*, Keelung; *KT*, Kenting; *GI*, Green Island.

identical (sequences matching 99% to 100% with DQ997879 + others), and formed a polytomy basal to *P. mutuki*. Very little (1-bp difference) or no variation in specimen sequences was seen within all subclades or groupings.

Within the *Zoanthus* + *Acrozoanthus* clade, four subclades were observed, one corresponding to *A. australiae* (ML = 61%, NJ = 79%; sequences matching 100% with AY995946), another to *Z. gigantus* (ML = 65%, NJ = 91%; sequences matching 100% with AB219193 + others), another to *Z. sansibaricus* (ML = 94%, NJ = 84%; sequences matching 100% with AB219188 + others), and another to *Z. kuroshio* and *Z. vietnamensis* (ML = 98%, NJ = 99%; *Z. vietnamensis* matching 100% with AB235409 + others). Within the *Z. kuroshio* + *Z. vietnamensis* subclade, all *Z. kuroshio* specimens formed a subclade (ML = 63%, NJ = 100%) with a 1-bp difference from *Z. vietnamensis,* matching 100% with previously reported *Z. kuroshio* sequences (e.g., AB235410 + others). Little (1 bp) or no variation in specimen sequences was seen within all *Zoanthus* + *Acrozoanthus* subclades.

Palythoa ITS-rDNA

ITS-rDNA sequences from collected *Palythoa* specimens all fit into one of the four large clades (Figure 3). Sequences from several specimens closely matched previously reported *P. heliodiscus* sequences (e.g., DQ997881), and formed a well-supported clade (ML = 100%, NJ = 100%). Some small variation was seen in sequences within this clade, notably a 35-bp insertion in the ITS-1 region of specimen YI2.

Sequences from three specimens (YI8, 1597, and 1635) formed a moderately well-supported clade (ML = 74%, NJ = 100%) with previously reported sequences of *Palythoa* sp. "sakurajimensis" from Japan (DQ997887).

Sequences from *P. mutuki* and *P. tuberculosa* formed large, moderately supported clades (ML = 83%, NJ = 98% and ML = 53%, NJ = 100% for NJ, respectively), and much variation in sequences was seen in both clades.

Zoanthus + *Acrozoanthus* ITS-rDNA

ITS-rDNA sequences were only obtainable for a few of all collected specimens in this group, with many other PCR products having double peaks, consistent with high amounts of variation previously reported in this group (Reimer et al. 2007d). For the BLAST results, sequences from specimens TF10, 1625, and 1636 matched 96% to 99% with previously reported sequences of *Z. gigantus* (e.g., DQ442438), those from specimens TF9 and 1632 matched 84% to 99% with previously reported sequences of *Z. sansibaricus* (AB214215 and AB214156, respectively), and those from specimen 1627 matched 97% with previously reported sequences of *Z. vietnamensis* (DQ442470). No usable sequences from *A. australiae* were obtained.

Symbiodinium ITS-rDNA

Within the generated ML tree, three major groupings were observed (Figure 4). The largest grouping consisted of subclade C1 and related derived sequences, and included ITS2 sequences from specimens 1586, 1595, 1599, 1617, 1619, 1620, 1621, 1622, 1625, 1630, 1631, 1633, 1635, 1640, 1641, 1642, 1658, 1661, 1669, TF8, YI8, YI14, GIGW1 to 5, PWHA1 to 4, TWLK 1 to 11, WLT1 to 15, and previously reported sequences from *Palythoa* and *Isaurus* species from the Pacific and Atlantic. Subclades c67, c69, c33, and c39 were also within this group, which had low bootstrap support (ML = 51%, NJ ≤ 50%). Within this group, one distinct subgrouping was observed, made up of four ITS2 sequences from Penghu *P. tuberculosa* specimens (PWHA9g1 to g4). This subclade had very high bootstrap support (ML = 98%, NJ = 100%).

Another grouping within the ML tree corresponded to subclade C3, and was basal to subclade C1. Within this grouping were subclades C22, C31, and C49, previously reported sequences from *P. tuberculosa* from Ishigaki, Japan, and sequences from specimens 1598, 1627, 1654, 1667, 1670, TF12, TF15, TF16, and YI2. There was also one subclade within this grouping, formed by sequences from 1632 and previously reported sequences from *Z. sansibaricus*.

The third major grouping consisted of sequences identical or closely related to sequence AB207184 from *Symbiodinium* in *Z. kuroshio* (= C15/C91-related). Sequences from specimens 1594, 1655, 1657, 1662, TF14, and YI1 were identical to AB207184, while ITS2 sequences from 1639 and YI24 formed a poorly supported separate clade (ML = 50%, NJ ≤ 50%). Two sequences from *Z. vietnamensis* in Keelung (1638 and 1639) formed another subclade with high support (ML = 90%, NJ = 86%).

Discussion

Zoanthid species identities and comparisons with previous morphological identifications

Results from the phylogenetic analyses of zoanthid specimens in this study generally confirm conclusions previously reached by Reimer et al. (2011b). Identifications in the previous study of specimens utilizing morphological features corresponded to phylogenetic species groupings observed in this study in almost all cases. In particular, results for specimens of *Z. sansibaricus*, *Z. gigantus*, *Z. kuroshio*, *A. australiae* (also reported in Reimer et al. 2011a), *P. heliodiscus*, *P. tuberculosa*, and *P. mutuki* matched the 2011 identifications. As previously reported (Reimer et al. 2006c, 2007c), *P. mutuki* may consist of more than one species, and evidence for this can be seen in the mt 16S rDNA tree with two separate groupings in this species, and in the large variety within the species' ITS-rDNA clade. *Palythoa tuberculosa* ITS-rDNA sequences from

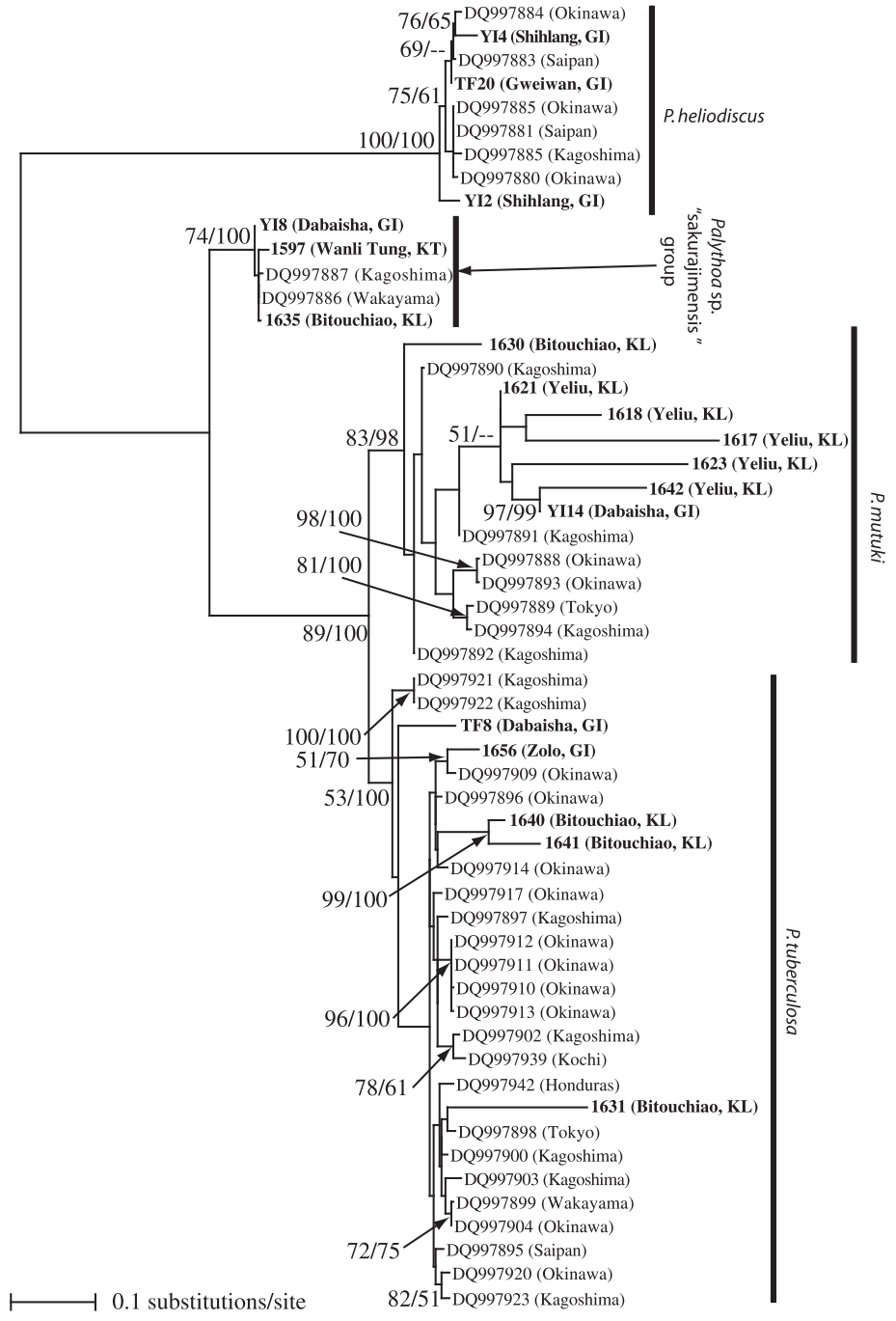

Figure 3 Maximum-likelihood (ML) trees of nuclear internal transcribed spacer region of ribosomal DNA (ITS-rDNA) sequences from *Palythoas* spp. Novel sequences from this study with specimen numbers and sampling locations are in boldface (species, specimen number). Sequences (GenBank accession number) from previous studies are in regular font. Values at nodes respectively represent ML and neighbor-joining (NJ) values. *KL*, Keelung; *KT*, Kenting; *GI*, Green Island.

Taiwan were often distinct from those of Japanese specimens but formed multiple clades. Given previous reports on *P. tuberculosa* (Reimer et al. 2007c) showing that this species has large amounts of ITS-rDNA variation, we feel that the explanation that this is one species is much more likely than the alternate hypothesis that there are many species in Taiwan (and Japan and the Indo-Pacific) with overlapping morphology and distributions.

One important finding from this study was the confirmation of subtle phylogenetic differences between *Z. kuroshio* and *Z. vietnamensis* (referred to as *Z.* aff. *vietnamensis* in Reimer et al. 2011b). It is known that

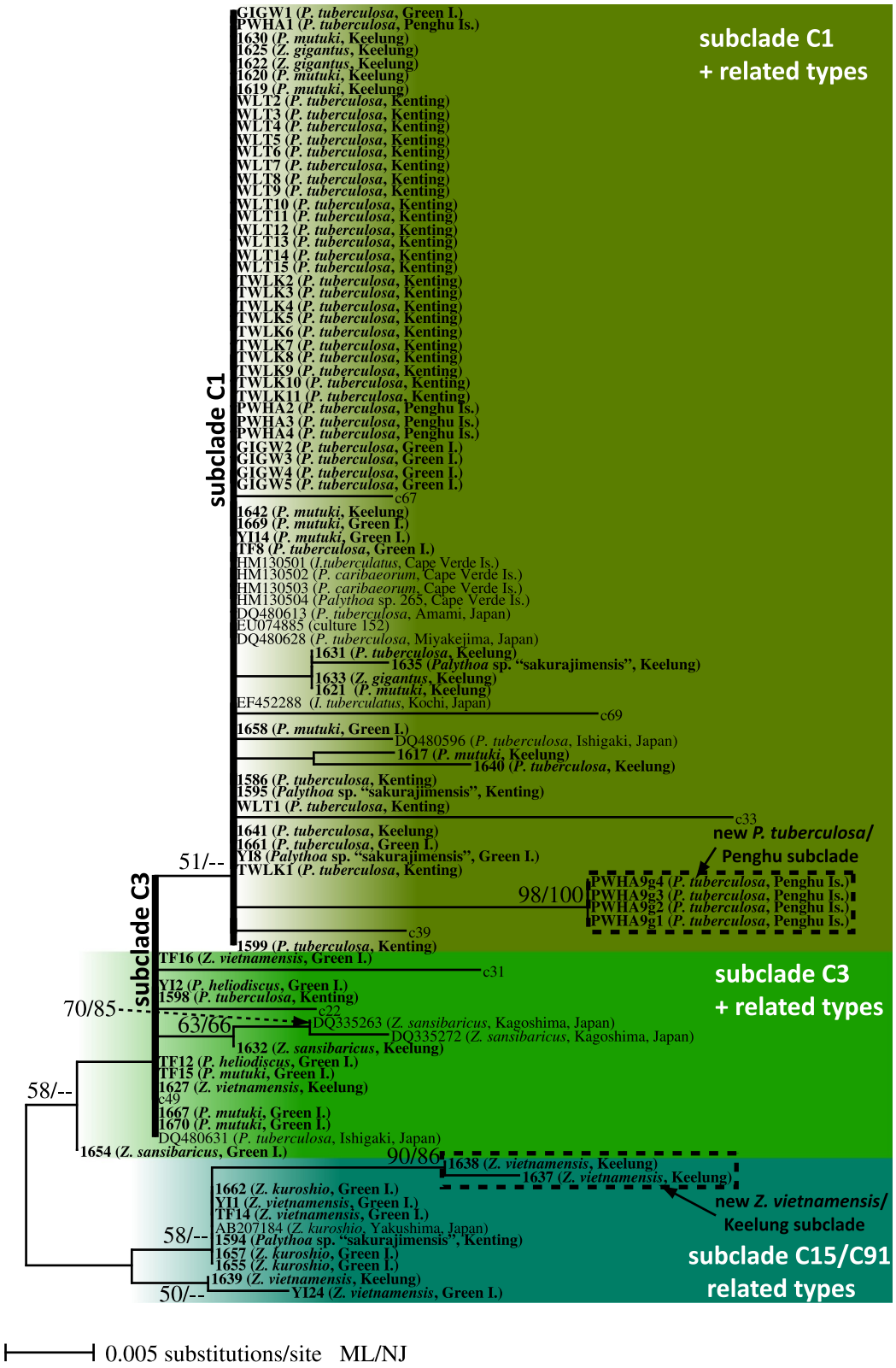

Figure 4 (See legend on next page.)

(See figure on previous page.)
Figure 4 Maximum-likelihood (ML) tree of internal transcribed spacer 2 (ITS2) sequences of *Symbiodinium* from zoanthid specimens from Taiwan. Novel sequences from this study with specimen numbers (see Table 1), host species names, and sampling regions are in boldface. Subclades C1 and C3 (common Pacific types) are designated by thick vertical lines. Sequences from previous studies are included with GenBank accession numbers and/or subclade names (sensu LaJeunesse 2001). Potential new novel subclades are designated by dashed boxes. Values at nodes respectively represent ML and neighbor-joining (NJ) bootstrap values.

these two species are phylogenetically very closely related, and the status of *Z. kuroshio* as a valid independent species has been in question (Reimer et al. 2006b). However, results from this study confirm the separation of these two species. A small phylogenetic difference (1 to 2 bp in mt 16S rDNA + COI sequences combined) was shown to be adequate to separate other zoanthid species (e.g., *P. tuberculosa* and *P. mutuki*) due to the slow rate of mtDNA mutations in Anthozoa (Shearer et al. 2002; Huang et al. 2008), and given the obvious morphological differences between these two groups (reminiscent of *P. tuberculosa* and *P. mutuki*), it is almost certain that these are two separate species. Further evidence comes the distribution of these two species in Taiwan, with *Z. kuroshio* only observed in coral reef environments (Kenting and Green Island) and *Z. vietnamensis* appearing most common at Keelung, a non-reef location. This is similar to the species' distribution in Japan, with *Z. kuroshio* most commonly seen in the southern Ryukyu Islands, and *Z. vietnamensis* reported from colder, more northerly locations and very rarely from the Ryukyus (Reimer 2010). *Z. vietnamensis* was described from Vietnam (Pax and Müller 1957) in the tropical South China Sea, but the coastline of Vietnam occasionally experiences winter sea surface temperatures of <20°C (Japan Oceanographic Data Center; JODC), and morphologically, the *Z. vietnamensis* specimens reported here agree well with the original description, while *Z. kuroshio* was described from the Ryukyu Archipelago, which is influenced by the warm Kuroshio Current, and the type locality of Yakushima also has low winter sea surface temperatures of approximately 20°C (JODC). *Zoanthus vietnamensis* may be a subtropical/temperate species, and *Z. kuroshio* a tropical/subtropical species, with some overlap in their distributions. Another possibility is that these two species prefer different environments, with the "immersae" (= polyps embedded in well-developed coenenchyme; Pax 1910) *Z. kuroshio* on exposed reef crests and reef slopes (Irei et al. 2011) and the "liberae" (= polyps free and clear of coenenchyme) *Z. vietnamensis* in more sheltered locations. However, no investigation into the habitat preference of *Z. vietnamensis* has been conducted, and therefore, research into the sexual reproduction, ecology, and distribution of these two species should help further clarify the differences between these closely related species.

One additional finding of this study is that there is likely more than one undescribed *Palythoa* species in the northwestern Pacific. Some specimens in this study were seen in the phylogenies to be closely related to undescribed *Palythoa* sp. "sakurajimensis", with other specimens previously reported from southern Japan (Reimer et al. 2007c) and the Ogasawara Islands (Reimer et al. 2011a). These specimens were initially identified in Reimer et al. (2011b) as either *P. mutuki* or *Palythoa* sp. In this study and in previous studies, *Palythoa* sp. "sakurajimensis" specimen numbers were low compared to other *Palythoa* species, and colonies consisted only of one or a few polyps. Distinguishing this putative species from *P. mutuki* based on morphology is currently very difficult, with *Palythoa* sp. "sakurajimensis" being found at slightly deeper depths than *P. mutuki*. A concerted effort to collect more *Palythoa* sp. "sakurajimensis" specimens from each region and their examination are needed to formally describe this species.

Another potentially undescribed species is represented by specimen YI2, which was identified as *P. heliodiscus* based on COI and mt 16S rDNA phylogenies, but it was previously identified as *Palythoa* sp. or *Palythoa* aff. *heliodiscus* based on morphology (Reimer et al. 2011b). A differing oral disc color (purple and/or green opposed to brown of *P. heliodiscus*) and ITS-rDNA results (Figure 3) indicate some differences between this specimen and *P. heliodiscus*. Similar insertions were seen in morphologically similar specimens from Singapore and Japan (P.A. Todd, T. Nishimura, and J.D. Reimer, unpublished data). As with *Palythoa* sp. "sakurajimensis", colonies of this putative species are likely to be rare, small, and cryptic.

For *Isaurus tuberculatus* reported in Reimer et al. (2011b), specimens were only observed by a third party and not collected, and no unforeseen results (e.g., undescribed species) are expected regarding their identity based on previous results (Muirhead and Ryland 1985; Reimer et al. 2008). Specimens of the "unknown zoanthid" from Reimer et al. (2011b) were also only observed but not collected, and these are very likely species within the recently described genus *Microzoanthus* Fujii & Reimer 2011, based on their extremely small size and morphological similarity to that genus.

Results of this study demonstrate that mt 16S rDNA sequences can more clearly define closely related species of zoanthids than can COI sequences. In this and previous

studies, COI sequences failed to resolve the following species groups from each other: *Z. vietnamensis* + *Z. kuroshio* and *P. mutuki* + *Palythoa* sp. "sakurajimensis". A COI sequence from one specimen of *P. mutuki* was also seen within the *P. tuberculosa* clade. Future phylogenetic work on brachycnemic zoanthids should always include mt 16S rDNA for this reason, and ITS-rDNA if possible.

Symbiodinium spp. diversity in zoanthids in Taiwan

Results of symbiont identification of Taiwanese zoanthids reflect other recent results reported from Japan, with some small but potentially important differences. Overall, association patterns as previously noted from different sites in Japan between various zoanthid host species and their symbionts were generally observed in this study. For example, the majority of Taiwanese *P. tuberculosa* (42 of 47 colonies or 89%) and *P. mutuki* (9 of 12 colonies or 75%) were associated with subclade C1 or closely related types, as reported in Japan (Reimer et al. 2006d). No *Palythoa* spp. were observed in association with clade D, which was reported from the Indian Ocean (Burnett 2002) and Singapore (Reimer and Todd 2009). *Symbiodinium* subclade C1 was shown to be a 'generalist' in terms of both associations and preferred environments, and together with subclade C3, is the most common Indo-Pacific type (LaJeunesse 2005). The lone *A. australiae* examined from Green Island was associated with clade D, as was previously reported from Kenting and Green Island (Reimer et al. 2010). Furthermore, *Z. sansibaricus* was associated with either C3-derived *Symbiodinium* (two of three colonies) or clade A (light tolerant) in shallow water, as was seen in Japan (Reimer et al. 2006e, 2007b). Finally, all three *Z. kuroshio* specimens examined had *Symbiodinium* ITS sequences identical to previously reported specimens from Japan (Reimer et al. 2006e) and Singapore (Reimer and Todd 2009).

However, some small differences from previous reports were seen. At Penghu, half (four of eight colonies) of *P. tuberculosa* specimens had a novel *Symbiodinium* C subclade derived from C1. This subclade had very high bootstrap support. These results suggest that *P. tuberculosa* can potentially associate with more than 1 clade C type. *Palythoa tuberculosa* is known to be a generalist species (Irei et al. 2011; Polak et al. 2011) with a high larval dispersal potential (Hirose et al. 2011; Polak et al. 2011), and associations with different symbiont types in different environments would seem to be a good strategy to adapt to different environments. However, while symbioses with clade D were observed in the Indian Ocean and Singapore, in the Pacific until now, *P. tuberculosa* is only known to associate with clade C1/C3 or very closely related types, even in remote oceanic locations such as the Ogasawara (Reimer et al. 2011a) and Galapagos Islands (Reimer and Hickman 2009). The marine environment of Penghu is quite different from Kenting and other subtropical and tropical areas in southeastern Taiwan, as winter water temperatures may dip to <15°C (Taiwan Central Weather Bureau), and often, the water is relatively turbid during the monsoon season (Huang et al. 2012a, b), and one or more of these factors may be contributed to the presence of this novel symbiont type. The occurrence of this divergent and novel type was somewhat unexpected, and further effort should be put into examining specimens from other areas near Penghu and the South China Sea, for which very few zoanthid data exist.

Another novel clade of symbionts was seen in *Z. vietnamensis* from Keelung. Additionally, it appeared that while *Z. kuroshio* from both Taiwan and Japan was associated only with one type of symbiont (C15/C91-derived, see Reimer et al. 2006e, 2007b), *Z. vietnamensis* is apparently more flexible in its association. Of the seven colonies examined in this study, two were associated with the new novel subclade (both at Keelung) and two with C15/C91 (identical to *Z. kuroshio*; both from Green Island), two were closely related to C15/C91 (Keelung/Green Island), and one was associated with subclade C3 (Keelung). Despite the examination of only seven colonies, the variety of types observed within *Z. vietnamensis* is surprising given its very close phylogenetic relationship with *Z. kuroshio* (Reimer et al. 2006b), and the fact that many other closely related zoanthid species are associated with similar or identical symbiont types, even in different oceans (e.g., Atlantic *P. caribaeroum,* sibling species to *P. tuberculosa*, harboring subclade C1 in Figure 1). Increasing specimen numbers with the inclusion of more specimens from the South China Sea (which is the type locality) and the Pacific coast of mainland Japan could also help clarify the nature of *Z. vietnamensis*' symbiont associations.

Finally, within this study, we examined zoanthid species for which no information on symbionts previously existed. *Z. gigantus*, *P. heliodiscus,* and *Palythoa* sp. "sakurajimensis" are not as common as the above mentioned zoanthid species (e.g., Irei et al. 2011) and are known to be distributed in both Taiwan and some locations in Japan (e.g., Reimer et al. 2011a). Specimen numbers of these species in this study are low (n = 2 to 4), and therefore, speculation on symbiont association patterns would be premature. The data acquired in this study should be compared to future, more in-depth studies. While all three of these species are usually associated with the "generalist" *Symbiodinium* C1 or C3 type, surprisingly, one *Palythoa* sp. "sakurajimensis" specimen from Kenting in southern Taiwan was associated with the C15/C91 type. This is the first time a *Palythoa* species has been reported to harbor this symbiont type. However, this species group contains multiple genotypes, and it is possible that it encompasses more than one species. Thus, any conclusions

on host-symbiont associations for this group should be treated with caution.

Conclusions

While preliminary morphological results from Reimer et al. (2011b) were accurate in identifying known zoanthid species, it appears that there are still undescribed *Palythoa* species present in the northwestern Pacific. These species may be widely distributed yet low in numbers, often cryptic in their habitat, and with a small colony size. Phylogenetic and molecular analyses would be extremely useful for finding such unknown groupings. Likewise, methods and DNA markers utilized in this study could be used to confirm if free-living *Sphenopus* (family Sphenopidae) zoanthids in Taiwan are two species or not as previously theorized (Soong et al. 1999).

Despite the large specimen numbers in this study, it is clear that much work remains to understand zoanthid-symbiont associations in Taiwan. Recently, *Symbiodinium* spp. examinations using more rapidly evolving markers such as the non-coding region of the chloroplast-encoded *psbA* minicircle demonstrated differences even for symbionts with identical ITS2 sequences (e.g., LaJeunesse and Thornhill 2011). Acquisition of additional sequence data and eventually the formal description of zoanthid-associated symbiont species following molecular methods outlined in LaJeunesse et al. (2012) will allow for more exact characterizations of symbionts of zoanthids. However, from the results of this and other recent studies, it is clear that different zoanthid species have differing patterns of association with *Symbiodinium*.

This study, combined with Reimer et al. (2011b), is intended to serve as a basis for the molecular phylogenetic study of shallow-water zoanthids and their symbionts in Taiwan.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JDR conceived the experiments, collected specimens, analyzed data, and wrote the paper. YI, TF, and SYY collected specimens, and carried out the molecular genetic studies. All authors read and approved the final manuscript.

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